Characterization of Holliday Structures in FLP Protein-Promoted Site-Specific Recombination

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Holliday structures are formed in the course of FLP protein-promoted site-specific recombination. Here, we demonstrate that Holliday structures are formed in reactions involving wild-type substrates and that they are kinetically competent with respect to the overall reaction rate. Together with a previous demonstration of chemical competence (L. Meyer-Leon, L.-C. Huang, S. W. Umlauf, M. M. Cox, and R. B. Inman, *Mol. Cell. Biol.* 8:3784-3796, 1988), Holliday structures therefore meet all criteria necessary to establish that they are obligate reaction intermediates in FLP-mediated site-specific recombination. In addition, kinetic evidence suggests that two distinct forms of the Holliday intermediate are present in the reaction pathway, interconverted in an isomerization process that is rate limiting at 0°C.

The FLP protein is a site-specific recombinase encoded by the 2- μ m plasmid of *Saccharomyces cerevisiae* (5). It belongs to the integrase family of site-specific recombinases, a family of eight known enzymes that includes the Cre protein of bacteriophage P1 and the Int protein of bacteriophage lambda (1, 6, 21, 22). These recombinases share a limited degree of homology at the amino acid level and some similarities in the architecture of their respective recognition sites.

The normal site of action (FLP recombination target [FRT]) of the FLP protein is a 48-base-pair (bp) DNA sequence that includes three 13-bp repeats arranged as shown in Fig. 1A, with an 8-bp spacer separating the first and second repeats. All three repeats are binding sites for FLP protein, although the third repeat can be deleted with no effect on in vitro recombination activity. The spacer has no direct FLP protein contacts within the central 6 bp (3, 25). The DNA is cleaved at the spacer boundaries, with FLP forming a 3'-phosphotyrosine intermediate (8). Sequence alterations in the spacer that maintain these sequence features are generally well tolerated by the system, with some constraints (26).

Two reacting FRT sites must have homologous sequences in the spacer. This permits productive alignment of nonpalindromic FRT sites from only one orientation (24). FRT sites with symmetrical spacers may align in either of the two possible orientations with respect to the flanking DNA and form recombination products. One substrate with a symmetrical spacer, pJFS39, reacts approximately as efficiently as its wild-type counterpart, pJFS36 (Fig. 1A) (24).

The FLP protein is stable and exhibits a slow catalytic turnover when glycerol and bovine serum albumin are added to the reaction mixture (7). Recombination also is observed when buffer and salt are the only solution additions besides DNA and protein (19), but inactivation of FLP under these conditions leads to an apparent stoichiometric requirement for this protein.

Recent studies have demonstrated that Holliday structures can be formed and/or resolved by the FLP, Cre, and Int proteins from substrates containing their respective

recombination sites (9, 11, 13, 14, 18, 20). (Holliday structures are defined as four DNA strands that are derived from two duplex DNAs and connected by a covalently closed joint [10]). All of these recent studies utilized either substrates bearing nicks or mutations in the recognition site or recombinases with amino acid substitutions. For instance, wild-type FLP protein efficiently formed stable Holliday structures from substrates containing symmetrical spacers and resolved them to products (18). It has proven difficult to detect and isolate Holliday structures from reactions involving a wild-type recombinase acting on its wild-type recombination site. Small amounts of Holliday structures have been observed in a normal FLP-mediated reaction by Jayaram et al. (13).

It is likely that Holliday structures are reaction intermediates in the site-specific recombination reactions promoted by integrase-class recombinases. However, isolation of a structure is only the first step in determining that it is an obligate reaction intermediate. Also required are demonstrations of (i) structure formation in reactions with unaltered substrates that involve wild-type enzyme, (ii) chemical competence, and (iii) kinetic competence. Chemical competence has previously been established for the FLP, Cre, and Int systems (9, 11, 18). Other characteristics are also consistent with the hypothesis that Holliday structures are recombination intermediates, including the fact that their steady-state concentration is proportional to the overall rate of reaction (18). Holliday structures also appear to be formed prior to or concurrent with the appearance of products (18).

The present study extends these results. Holliday structures were isolated from a completely wild-type recombination reaction under conditions conducive to efficient turnover, confirming the observations of Jayaram et al. (13). We further demonstrate that Holliday structures are kinetically competent in this reaction. Additional evidence suggests that there are at least two discrete Holliday structures on the reaction pathway, interconverted in a step that becomes rate limiting at 0°C.

MATERIALS AND METHODS

Materials. FLP protein (molecular weight of 48,794, on the basis of its amino acid sequence) was prepared by ion-exchange and affinity chromatography according to a previ-

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ously published procedure (17). Protein concentrations were determined by using the Bradford assay (2). The degree of purity was multiplied by the total protein concentration (0.148 mg/ml) to determine the molarity of FLP protein monomers (1.62 μ M). FLP protein used in this study constitutes approximately 85% of the total protein, on the basis of densitometric scanning of a polyacrylamide gel. DNA was purified by banding twice in ethidium bromide-cesium chloride gradients. DNA concentrations (in total nucleotides)

were measured at 260 nm, assuming that 1.0 absorption unit was equivalent to 50 μ g/ml or 149 μ M. Restriction enzymes were purchased from New England BioLabs. All other reagents were of analytical grade and were purchased from other vendors.

Substrates. Plasmids of the pJFS series were constructed by inserting an FRT site into the plasmid pXF3 (16) between the *Eco*RI and *Bam*HI restriction sites. All of these plasmids are identical except for alterations to the FRT site, as noted in Fig. 1A. pJFS36 (2,822 bp) (23), pJFS35r (2,820 bp) (23), and pJFS39 (2,821 bp) (24) have been described previously. The plasmid pJFS61 (2,835 bp) was constructed by digesting pJFS36 and pJFS122 (23) with *Pst*I and *Xba*I and subsequently ligating the small *Pst*I-*Xba*I fragment of pJFS36 to the large *Pst*I-*Xba*I fragment of pJFS122. This reconstructed the intact wild-type FRT site between the *Eco*RI and *Bam*HI sites of pXF3 (J. Senecoff and M. Cox, unpublished results).

The pJFS plasmids contain unique *Pst*I and *Sal*I restriction sites (Fig. 1). All recombination reactions reported in this study were intermolecular and were performed with plasmid substrates linearized at one of two restriction sites: pJFS61 and pJFS36 at the *Sal*I site and pJFS39 and pJFS35r at the *Pst*I site. The plasmid pMMC10 (4) (6,530 bp) contains the complete wild-type FRT site within a 3,379-bp segment of 2 μ m plasmid DNA. Reactions with this substrate used a plasmid linearized at a unique *Bam*HI site. Restriction endonuclease digestions were performed under conditions recommended by New England BioLabs. Digestion with each of these restriction enzymes positions the FRT site at least 276 bp from the end of the linear DNA. Except for pJFS39, all spacers are asymmetrical and reaction occurs only when FRT sites are aligned in the same orientation (see above for explanation). For pJFS39, which has a symmetrical spacer, reactions can occur with FRT sites aligned in either orientation (24). A parallel reaction is defined as that orientation in which homologous flanking sequences are aligned on the same side of the FRT site. An antiparallel reaction results from the opposite alignment, in which corresponding flanking sequences are at opposite sides of the FRT site. The sequence differences between these FRT sites are presented in Fig. 1A.

Each FRT site contains two FLP protein-binding sites (13-bp inverted repeats), except for pJFS61 and pMMC10, which have the normal three 13-bp inverted repeats per FRT site. Each substrate plasmid used in this study contains a single FRT site, and substrate DNA concentrations are reported in terms of total FRT site concentrations so that the FLP protein/FRT stoichiometry is readily apparent. The concentration of FRT sites is simply the DNA concentration (in nucleotides) divided by the length of the DNA substrate in question. We have found that DNA flanking the FRT sites

FIG. 1. Diagram of FLP protein-mediated recombination reactions described in this report. (A) FRT sites used in this study. The minimally required recombination site (FRT) contains a spacer region with two flanking inverted repeats. The spacer sequence of each FRT site is shown, and the repeat sequences (13 bp) are denoted by arrows. The spacer is normally 8 bp long. The decrease to 7 bp in the pJFS35r spacer results in a small decrease in recombination efficiency (24). The wild-type site (found in pMMC10 and pJFS61) contains a third repeat sequence. (B) Recombination between two *Pst*I-digested pJFS39 molecules, which contain a symmetrical spacer, can occur in a parallel or antiparallel orientation. The antiparallel reaction results in discrete products of 1.6 and 4.0 kilobase pairs (kb). (C) Recombination between *Bam*HI-digested pMMC10 and *Sal*I-digested pJFS61 results in products of 5.0 and 4.3 kb. (D) Recombination between *Sal*I-digested pJFS36 and a *Pst*I-

digested pJFS plasmid results in discrete products of 4.5 and 1.1 kb. In each of the reactions illustrated (panels B, C, and D), recombination between two identical plasmids (cut with the same restriction enzyme) in the same orientation results in products of the same size and with the same gel mobility as that of the substrates (data not shown). In most cases, these unobserved reactions represent one-half of the total. The letters a, b, c, d, e, and f specify the distance in kilobase pairs (numbers are rounded off for convenience) between the FRT site and the unique restriction site used to linearize the molecule, where a = 0.8 kb, b = 2.0 kb, c = 2.5 kb, d = 0.3 kb, e = 4.0 kb, and f = 2.5 kb. Sequences a to d are pXF3-derived sequences; e and f are yeast 2 μ m plasmid-derived sequences. All FRT sites used in this study contain an *Xba*I site within the spacer. P, *Pst*I site; S, *Sal*I site; B, *Bam*HI site; ■, FRT site.

does not detectably affect reaction rates over the range of DNA lengths used in this study (C. Ullsperger, L. Meyer-Leon, and M. Cox, unpublished results). The terms FRT and substrate are used interchangeably in the text.

Isolation of Holliday structures. DNA was isolated prior to electron microscopy by using an IBI Unidirectional Electroeluter (*Life on the Edge*, vol. 2, p. 41–43, International Biotechnologies, Inc., New Haven, Conn., 1986/1987) as previously described (18). To prepare Holliday structures for kinetic experiments, DNA was extracted from excised gel bands with GeneClean (Bio101 Inc., La Jolla, Calif.). The concentration of DNA (total nucleotides) in Holliday structure preparations was determined by analyzing the A_{260} of the solution as described above.

Reaction conditions and quantitation. All reaction mixtures (10 μ l) contained 25 mM TAPS {3-[*N*-tris(hydroxymethyl)methylamino]propane sulfonic acid} (pH 8.0); 1 mM EDTA; 0.2 M NaCl; 10% (wt/vol) polyethylene glycol, molecular weight 8,000; 20% glycerol; 2.5 mg of bovine serum albumin per ml; and variable amounts of substrate DNA and FLP protein, as described in the text. For time courses, proportionally larger reaction volumes were employed, and 10- μ l portions were removed at each time point. All reaction constituents except FLP protein were mixed and preincubated at the appropriate reaction temperature (30°C or 0°C) for 5 min before initiating the reaction with FLP protein. Incubations at 0°C were performed on ice. The portion labeled zero time was removed just before the reaction with FLP protein was begun. Reactions were terminated with 2 μ l of 5% sodium dodecyl sulfate in a solution of xylene cyanole and bromophenol blue in H₂O. Reactions were analyzed by using 1.0 or 1.5% agarose gels that had been run in TAE buffer (40 mM Tris acetate, 1 mM EDTA), stained with ethidium bromide, and photographed with Polaroid type 55 film. This film produces both positive and negative photographic images.

The progress of a site-specific recombination reaction was quantitated by densitometric scanning of the photographic negative by using a Zeineh Soft Laser scanning densitometer (model SL-504-XL). All kinetics experiments were carried out with substrates and isolated Holliday structures derived from pJFS39. The two discrete linear products observed in a typical reaction are denoted P1 (larger than pJFS39, 4,096 bp) and P2 (the smaller product, 1,546 bp) (Fig. 1B).

The relative amount of DNA represented by each band observed on an agarose gel was calculated in the following manner. The scanning intensity, measured in integrative units, of the substrate (I_{U_s}), the large product (I_{U_p}), and the intermediate (I_{U_x}) bands was normalized to compare the number of DNA molecules in each gel band. This is necessary because the amount of ethidium bromide bound by each DNA species, and thus the intensity of the signal contributed by each DNA molecule, is largely a function of DNA length. I_{U_x} was multiplied by 0.5 since there are two substrate equivalents per Holliday structure, and I_{U_p} was multiplied by 4,096/2,821, or 0.69; therefore, $[S] = (I_{U_s})1$, $[P1] = (I_{U_p})0.69$, and $[X] = (I_{U_x})0.5$. Since P1 and P2 are always produced in equal amounts, only P1 was monitored and the number of FRT sites in the P1 product was multiplied by 2 to account for the unmeasured P2 product: $[P] = [P1] + [P2] = 2[P1]$. The total concentration of FRT sites is as follows: $[FRT] = [S] + [P] + 2[X]$; therefore, $\%P = [P]/[FRT]$. Similarly, percent X equals $2[X]/[FRT]$, when expressed as nanomolar FRT sites, or $[X]/[FRT]$, when expressed as nanomolar Holliday structures. In some cases, the fraction of FRT sites in a particular DNA band was multiplied by the

known concentration of FRT sites in the sample to convert to the molar scale. Initial rates of product formation were estimated from the slopes of lines drawn through early points in reaction progress curves. The apparent k_{cat} was obtained by dividing the initial rate by the concentration of FLP protein monomers. In comparative trials, this method of correcting for inherent differences in the signal intensity produced by different DNA species and calculating the rate of product formation provided results very similar to those obtained by using the methods of Gates and Cox (7). The degree of variation in measured reaction rates observed in different experiments is described in the text.

Holliday structure resolution (see Fig. 4B) was quantitated by subtracting the concentration of intact Holliday structures at each time point (X_t) from the concentration of intact Holliday structures present at zero time (X_0). A portion of the reaction mixture was removed just before the addition of FLP protein, and this portion was used to determine the concentration of the isolated Holliday structures at zero time. Typically, 60 to 90% of the total FRT sites were present in the Holliday structure band at zero time, as determined by densitometric scanning (described above). The remaining FRT sites were present in the linear substrate, and product DNAs were present to some degree in all Holliday structure isolates. The concentration of FRT sites in the intact Holliday structure is the total FRT site concentration added to the reaction (total nucleotide concentration divided by 2,821) minus the number of FRT sites measured in the product and substrate bands. Holliday structure concentrations in kinetic experiments are expressed in terms of total FRT sites to facilitate comparison with kinetic data from normal recombination reactions. The apparent k_{cat} of these reactions was calculated in terms of FLP protein monomers and corrected for the fraction of FRT sites not present in Holliday structures at zero time, as described in the text.

Electron microscopy. Samples were prepared for electron microscopy by a modification of the cytochrome *c* spreading technique, in which DNA is spread onto a small water drop (12), as previously described (18). No attempt was made to preserve DNA-protein structures; consequently, any protein present was denatured and incorporated into the cytochrome *c* supporting monolayer.

Computer-assisted length measurements were made from electron monographs by tracing and recording on a semiautomatic digitizer, as described previously (15). All measured lengths were calculated in base pairs, relative to substrate lengths of 2,835 (pJFS61) and 6,530 bp (pMMC10).

RESULTS

The first goal of this study was to show that Holliday structures could be readily isolated from FLP recombination reactions by using a wild-type FRT site. Our previous attempts at this made use of the substrate pJFS36 (18), which contains a wild-type FRT site, with the exception that the distal 13-bp repeat is deleted (Fig. 1A). Only parallel site alignment results in product formation. Therefore, the FRT sites within a pJFS36 $\times$ pJFS36 Holliday structure are flanked by homologous sequences extending to the ends of the DNAs. Because the resulting Holliday junction is free to branch migrate, the Holliday structures are preparatively unstable and few are seen (18).

In order to stabilize Holliday structures for analysis and isolation, it was necessary to use two DNA substrates in which the FRT sites were flanked by heterologous DNA.

This was achieved in two ways. The first approach, which was successfully employed previously (18) and was further exploited in this study, was to use an FRT site with a symmetrical spacer, pJFS39 (Fig. 1B). Because of their symmetry, the FRT sites were capable of recombining after either a parallel or an antiparallel alignment. The Holliday structures resulting from a parallel reaction were not observed as a result of elimination by branch migration of the Holliday junction (18). When site alignment was antiparallel, however, the Holliday junction was prevented from migrating outside the FRT site by heterologous sequences in the flanking DNA and was therefore stable. Those Holliday structures observed on an agarose gel of a pJFS39 reaction all result from the antiparallel reaction (18).

The second approach was to insert the wild-type FRT site, with all three repeats, into two different vectors, pMMC10 and pJFS61. The FRT sites were flanked by pBR322 DNA on pJFS61 and by 2- μ m plasmid DNA on pMMC10. In a reaction between pMMC10 and pJFS61 the Holliday junction was not able to migrate outside the FRT site, conserving the Holliday structure. The products formed in this reaction differed in size from the substrates (Fig. 1C) and were detectable in the agarose gel assay. In both types of reaction (Fig. 1B and C), half of the recombination events were between substrates of identical size and composition, in turn yielding products of equal size that could not be observed by gel electrophoresis. Therefore, a maximal reaction was one in which 50% of the substrates were converted to discrete products. The spacer sequences of the substrates used in this study are shown in Fig. 1A.

Holliday structures are formed from wild-type substrates. A time course of an FLP recombination reaction with *Pst*I-cut pJFS39, a substrate containing a symmetrical spacer, is shown in Fig. 2, lanes 1 to 7. Since the observed concentration of Holliday structures is a function of FLP protein concentration at 30°C (18), a large excess of FLP protein was used in this experiment. Holliday structures are at an approximate steady-state level at the first measurable time point, 20 s, and remain so over most of the duration of the reaction. The observed Holliday structures result from the antiparallel recombination reaction, as noted above. To determine whether Holliday structures were present in the wild-type reaction, a similar experiment was performed with pMMC10 and pJFS61 and is shown in lanes 8 to 14. This reaction produces the expected products and substrate-sized bands. In addition, bands of slower electrophoretic mobility are visible at the top of the gel, one of which is labeled X. This band was excised from the agarose and examined by electron microscopy (Fig. 3). When spread, 74% of 147 molecules appeared to be characteristic Holliday structures. Most of the remaining DNA was composed of linear DNA molecules, which we assume arose from DNA breakage during handling or from background in the gel lane. The four DNA arms of 17 Holliday structures were measured and found to be 3,970, 2,570, 2,530, and 300 bp. This corresponded, within the experimental error of ± 35 bp, with the predicted arm lengths of 3,984, 2,547, 2,532, and 303 bp and confirmed that the junction of the Holliday structure is site specific and located at the FRT site. These results are consistent with Holliday structures characterized previously (13, 18) and show that Holliday structures can be isolated from the wild-type FLP recombination reaction. As would be expected, Holliday structures resulting from the pMMC10 $\times$ pMMC10 or pJFS61 $\times$ pJFS61 reaction were not observed. This is probably the result of instability of the

FIG. 2. Time courses showing the steady-state concentration of Holliday structures during recombination reactions with the substrate pJFS39 (lanes 1 to 7) and with the wild-type substrates pJFS61 and pMMC10 (lanes 8 to 14). Portions were withdrawn from each reaction at 0, 0.33, 1.0, 2.0, 5.0, 10, and 30 min after initiating the reaction with FLP protein. These are displayed left to right on the gel for each reaction. In each reaction, FRT sites are present at 14.2 nM and FLP protein is present at 271 nM, a ratio of FLP monomers per FRT site of 19:1. Labels on the left refer to the pJFS39 reaction; those on the right refer to the wild-type reaction. X, Holliday structure; S, substrate; P1 and P2, large and small products, respectively; D, double-strand cleavage product. The larger substrate in lanes 8 to 14 is pMMC10. Bands visible above band X in lanes 8 to 14 are uncharacterized reaction by-products (18). The reactions were carried out at 30°C.

junction due to branch migration, as has been previously described (18).

The concentration of Holliday structures in the wild-type reaction (Fig. 2, lanes 8 to 14) also appeared to remain at steady state throughout the reaction. To determine whether the steady-state concentration was the same in reactions of substrates containing symmetric spacers as in that containing asymmetric wild-type spacers, the reactions were quantitated by laser densitometry. The concentration of FRT sites in the form of Holliday structures was calculated as a percentage of the total FRT sites in the reaction mixture. On average, 12.9% of the total FRT sites were present in Holliday structures over the time period 1 to 10 min in the pJFS39 reaction and 3.1% were present in the wild-type reaction. Thus, under these conditions there is a fourfold increase in the observed steady-state level of Holliday structures when a substrate with a symmetric spacer is used compared with the wild-type. In both reactions, the observed Holliday structures represent only half of the actual Holliday structures formed during recombination, as explained above.

Kinetic competence of the Holliday structure. To show that the Holliday structures are kinetically competent reaction intermediates, they were isolated and their rate of resolution was compared with that of the overall rate of reaction. First, the overall rate of conversion of pJFS39 substrate to product was calculated under reaction conditions that supported enzyme turnover and included 2.7 nM FLP protein mono-

FIG. 3. Holliday structure as revealed by electron microscopy. This molecule was isolated from an FLP recombination reaction between the substrates pJFS61 and pMMC10 carried out at 30°C. Both plasmids contain the wild-type FRT site, but they differ in the vector sequences flanking the site. The measurement of 17 such molecules yielded arm lengths of 3,970, 300, 2,530, and 2,570 bp, corresponding to arms arranged clockwise from the longest arm.

mers. The time courses were repeated many times with different concentrations of *Pst*I-cut pJFS39 substrate DNA. One such time course, at 5.4 nM FRT site, is shown in Fig. 4A. From the initial rate of reaction (0.125 FRT sites per min), an apparent k_{cat} (v/E) of $\sim 0.046 \text{ min}^{-1}$ was calculated. Repeated assays produced a range of k_{cat} values from 0.041 to 0.055 min^{-1} . Higher substrate concentrations, 10.8 and 21 nM, yielded similar values for v/E , indicating that the system is at or near substrate saturation and that this is a valid measure of k_{cat} . While the k_{cat} value measured in this study is somewhat lower than that previously reported (0.12 min^{-1} [7]), values have been consistent throughout this set of experiments. The differences probably result from the use of different enzyme preparations in the two sets of experiments. The enzyme used previously was purified by a different protocol (C. Gates and P. Rossmeyss, unpublished results), and we do not have an accurate estimate of the amount of inactive enzyme, if any, contained in either preparation.

To determine the rate of Holliday structure resolution, Holliday structures were purified from an agarose gel as described in Materials and Methods and reacted with fresh FLP protein (2.7 nM) under the same conditions. The total concentration of FRT sites in the reaction (5.4 nM) was identical to that used in the recombination reaction de-

FIG. 4. Rate of Holliday structure resolution versus the overall rate of reaction at 30°C. (A) Plot of observed product formation versus time in a recombination reaction involving pJFS39 (Fig. 1B), with data expressed in terms of nanomolar FRT sites. (B) Time course of the resolution of Holliday structures isolated from a pJFS39 reaction. The concentration of Holliday structures is expressed in terms of total FRT sites to facilitate comparison with the reaction in panel A. In both reactions, the total concentration of FRT sites is 5.4 nM and the FLP protein concentration is 2.7 nM. In panel B, 62.5% (3.37 nM) of the FRT sites are present in Holliday structures. The horizontal dashed lines in panels A and B represent the theoretical maximum reaction possible in each experiment. The sloped dashed lines were used to estimate initial reaction rates. In panel A, the observed initial rate is 0.0625 nM FRT sites per min, or 0.125 nM/min when the unobserved recombination events are included. In panel B, the observed initial rate is 0.09 nM FRT sites per min before correction as described in the text.

scribed above. The FRT sites present in intact Holliday structures represented 62.5% of the total, or 3.37 nM at zero time. A time course was performed (Fig. 4B), and the initial rate of Holliday structure resolution was 0.09 nM FRT sites per min, translating into an apparent k_{cat} of 0.033 min^{-1} . Assuming that all of the FRT sites in the reaction mixture at zero time ($P + S + X$) are equivalent with respect to likely interaction with FLP protein, only 62.5% of the FLP protein, or 1.69 nM, are interacting with Holliday structures at early times. This corrected FLP protein concentration gives an apparent k_{cat} of 0.053 min^{-1} for the resolution of Holliday intermediates. This turnover value is consistent with the apparent turnover rate of the recombination reaction under these conditions and confirms that the Holliday structure is resolved by FLP protein to recombinant product at rates

FIG. 5. Comparison of the rate of Holliday structure formation with the rate of product formation with two different substrates. Lanes: 1 to 9, time course showing a reaction of *Pst*I-digested pJFS39; 10 to 18, time course showing a reaction of *Pst*I-digested pJFS35r $\times$ *Sal*I-digested pJFS36. For both reactions, samples were withdrawn at 0, 0.25, 0.5, 1.0, 2.0, 5.0, 10, 30, and 60 min (left to right). The total concentration of FRT sites in each reaction was 21.5 nM. FLP protein was present at 325 nM, a ratio of 15 FLP monomers per FRT site. The upper band of the doublet represented by X in the pJFS35r $\times$ pJFS36 reaction is a three-arm molecule resulting from branch migration of the Holliday junction to the ends of the linear molecule, as previously described (18). X, Holliday structure; S, substrate; P1 and P2, large and small products, respectively; D, double-strand cleavage product. Labels to the left refer to the pJFS39 reaction, and those to the right refer to the pJFS36 $\times$ pJFS35r reaction. Reactions were carried out at 30°C.

comparable to the overall rate of reaction. This experiment was repeated several times with different Holliday structure preparations, yielding uncorrected k_{cat} values in the range of 0.03 to 0.06 min⁻¹. The curvature seen in this plot was consistent with that seen in other experiments and probably reflects either a back reaction or a sequestering of FLP protein in reactions with FRT sites in the S and P DNA species as the concentrations of S and P increase.

Holliday structure formation precedes product formation. Under reaction conditions in which FLP protein is slightly in excess (relative to the number of available binding sites in FRT sites), the Holliday structure forms only slightly faster than product does (for example, see Fig. 2, lane 2). It appears to be converted to product rapidly. However, at lower enzyme concentrations, the level of Holliday structure is too low to be clearly visible. Therefore, Holliday structures have not been observed in the complete absence of products under standard reaction conditions. To show more clearly that Holliday structure formation preceded product formation, we sought strategies that would slow the rate of resolution of the intermediate. Two such strategies were found: (i) the use of substrates with spacer mismatches and (ii) low-temperature conditions.

The first strategy made use of the substrate pJFS35r, which has a 1-bp deletion in the spacer (Fig. 1A). This substrate recombines efficiently when reacted with itself (pJFS35r $\times$ pJFS35r) but is very inefficient when reacted with a substrate containing a wild-type spacer (pJFS35r $\times$

FIG. 6. Rate of Holliday structure and product formation at 30°C versus that at 0°C. Agarose gel showing time courses of *Pst*I-digested pJFS39 at 30°C (lanes 1 to 10) or 0°C (lanes 11 to 20). Portions were removed from each reaction at 0, 0.5, 1.0, 2.0, 5.0, 10, 30, 60, 120, and 480 min (left to right in each series). X, Holliday structure; S, substrate; P1 and P2, large and small products, respectively; D, double-strand cleavage product. The concentration of FRT sites in each reaction was 10.8 nM, and that of FLP protein was 60 nM, a ratio of 6 FLP monomers per FRT site. Bands visible above the X band are uncharacterized reaction by-products (18).

pJFS36) (23). Holliday structures are observed in the latter reaction, however (18). Figure 5 shows a comparison between a reaction of *Pst*I-cut pJFS39 (lanes 1 to 9) and a reaction of *Sal*I-cut pJFS36 $\times$ *Pst*I-cut pJFS35r (lanes 10 to 18). The initial rate of Holliday structure formation is approximately the same in both reactions. However, the steady-state accumulation of Holliday structures was almost twofold greater for the pJFS35r $\times$ pJFS36 reaction, and product formation was 77-fold slower. Conversion of the Holliday structure to product is clearly rate determining under conditions in which there is a 1-bp deletion in the spacer of one reacting partner.

The rate of product formation was also decreased by lowering the reaction temperature to 0°C. Figure 6 shows two time courses with the pJFS39 substrate. The reaction on the left (lanes 1 to 10) was carried out at 30°C, and the time course to the right was done at 0°C (lanes 11 to 20). The rate of formation of Holliday structures is similar at both temperatures. However, the rate of product formation is 16-fold higher at 30°C than at 0°C, suggesting that the decreased temperature affects the conversion of Holliday structures to products much more than it affects their formation. Both of the reactions shown in Fig. 6 included 10.8 nM FRT sites and 80 nM FLP protein, a ratio of 7.5 FLP protein monomers per FRT site.

Time courses were also performed at 0°C by fixing the FRT site concentration at 10.8 nM but varying the FLP protein concentration between 5 and 200 nM (Fig. 7). At FLP protein concentrations equal to or greater than 20 nM, increasing the FLP protein concentration did not alter the observed concentration of Holliday structure outside the experimental error. The apparent rate of Holliday structure

FIG. 7. Percent of FRT sites in Holliday structures at steady state versus the FLP protein concentration. Each point is the mean measured level from six time points taken at 5, 10, 30, 60, 120, and 240 min. The level of Holliday intermediate is at an apparent steady state throughout this time span. Error bars represent the sampling error at a 95% confidence level. Percentages are calculated as the concentration of FRT sites in Holliday structures divided by the total FRT site concentration. There are two FRT sites per Holliday structure, and so the fraction of DNA species that are Holliday structures is approximately $\frac{1}{2}$ of that shown.

formation and the rate of product formation are also not affected by varying the enzyme levels above 20 nM (data not shown). At 0°C, therefore, both the concentration of Holliday intermediate and the rate of reaction exhibit saturation behavior with respect to the concentration of FLP protein. This is in contrast to the dependence of the observed steady-state level of Holliday structures on enzyme concentration seen at 30°C (18) and the strong dependence of reaction rate on FLP protein concentration seen at 30°C, even for protein concentrations well in excess of the substrate concentration (7). A step occurring between the formation of Holliday structures and the appearance of products becomes rate limiting at 0°C. The rate of this step is not affected by the concentration of FLP protein above concentrations required to saturate available FRT sites. In the Discussion, we present arguments that this step is an isomerization step that interconverts two distinct forms of the Holliday intermediate-FLP protein complex.

DISCUSSION

In this report, we continue to characterize Holliday structures formed by the FLP site-specific recombination system. The primary conclusions are as follows. (i) Holliday structures meet all criteria for reaction intermediates. (ii) There are at least two distinct Holliday intermediates on the reaction pathway, interconverted in a step that becomes rate limiting at 0°C.

Holliday structures are formed by wild-type FLP protein from the wild-type FRT site under reaction conditions that support enzymatic turnover. The steady-state concentration of Holliday structures derived from wild-type substrates is approximately fourfold lower than that of Holliday structures derived from a substrate with a symmetrical spacer. Isolated Holliday structures are resolved at approximately the same rate as the overall recombination reaction at 30°C. Because Holliday structures are also formed prior to recombination products, this shows that Holliday structures are kinetically competent as reaction intermediates. Together with the prior demonstration that these molecules are chem-

ically competent (18), we have shown that Holliday structures meet established criteria for defining reaction intermediates. This represents a significant step towards outlining a reaction pathway for FLP protein-promoted site-specific recombination.

The concentration of Holliday structures is lower in the wild-type reaction than it is with substrates with symmetrical spacers, because of slower formation, faster resolution, or both. The reason for this is not clear.

The model for strand exchange via a Holliday intermediate must involve several sequential reaction segments. After location by FLP protein of its recombination site and juxtaposition of two protein-bound DNAs, one strand of each partner is cleaved and religated to the analogous strand of the opposing substrate. This forms a Holliday intermediate. The junction must branch migrate across the 8-bp spacer, after which the second strand of each reacting partner is cleaved and religated in turn. This short branch migration might be part of an isomerization step within a complex of the Holliday intermediate and its associated protein. A change in protein conformation or a rearrangement of FLP subunits in the complex could be an implicit part of the isomerization step.

In two of the experiments reported here, Holliday structures were formed at nearly normal rates but resolved to products slowly. In a reaction between the substrate pJFS36 and another that had a 1-bp deletion in the spacer (pJFS35r), Holliday structures were formed at near-normal rates but the rate of product formation was 77-fold lower. The deletion may cause a structural perturbation in the Holliday junction that could affect either the cleavage reactions required for product formation or the isomerization step outlined above. Product formation is also very slow at 0°C, and here the results are more informative.

An argument can be developed from the low-temperature-experiment data that an isomerization is a kinetically significant process in the reaction pathway and that it is this step that becomes rate limiting at 0°C. Such a step would, in effect, interconvert two discrete Holliday intermediates. The argument proceeds from evidence that the covalent bond cleavage and formation (transesterification) steps taking place in the course of a site-specific recombination event are relatively fast. Phosphodiester bonds are always conserved in these reactions, and, in the FLP system, the overall reaction can be considered thermodynamically symmetrical. At 0°C, the transesterification steps do not appear to be rate limiting. First, the formation of a Holliday structure involves four separate transesterification steps, and these structures are formed nearly as rapidly at 0°C as at 30°C. Low temperature therefore has a minimal effect on the transesterification steps required to form Holliday structures. Second, and perhaps more important, is the observed effect of increasing FLP protein concentration (Fig. 7). At concentrations of FLP protein exceeding that required to bind all FRT sites, the rate of Holliday structure formation and the observed concentration of Holliday junctions formed at 0°C are independent of protein concentration. This suggests that substrate is truly saturated with FLP protein. It also suggests that the observed level of Holliday structures reflects an equilibrium with substrate under these conditions ($2S \rightleftharpoons X$, where S = substrate and X = Holliday structure). Finally, the set of transesterification reactions that produces the Holliday junction should be readily reversible. The covalent bonds broken and reformed are identical in both directions, $2S \rightarrow X$ and $X \rightarrow 2S$. The equivalent set of transesterification reactions required to convert Holliday structures to products ($X \rightarrow 2P$) must be equally fast. By this reasoning, the slow

step in product formation at 0°C is almost certainly not a step involving the cleavage or formation of phosphodiester bonds. The only step remaining that would explain the slow product formation, therefore, is a molecular isomerization that moves the crossover point from the first set of cleavage sites across the spacer to the second.

The isomerization/branch migration step would be expected to have a high temperature coefficient and to be affected by heterology in the spacer, and the step that limits product formation at 0°C clearly has these characteristics. In addition, mutations that increase the A+T content of the central 6 bp of the spacer, a change that could be expected to facilitate branch migration, increase the rate of product formation at 0°C without affecting the production of Holliday structures (L. Waite and M. Cox, unpublished data). It is important, however, not to draw too close an analogy between this process and the spontaneous branch migration of cross-stranded structures observed and studied in solution. The Holliday structures in the FLP system are almost certainly protein bound and held in a precise conformation. The step here is likely to involve rearrangement of protein as well as DNA, making the term isomerization more appropriately descriptive than the term branch migration.

The implication is that there are at least two distinct Holliday structures on the reaction pathway, X1 and X2, and that their interconversion is kinetically significant and can be rate limiting. This pathway is illustrated by:

where S is the starting substrate and P is the final recombination product. At 30°C, the two forms of Holliday intermediate would probably interconvert rapidly because the barrier to isomerization would be low. Both the rate of product formation and the Holliday structure concentration are directly related to FLP protein concentration at this temperature (7, 18). This suggests that the rate-determining step at 30°C is not isomerization but a step that is dependent on the concentration of enzyme. The most likely candidates are site location and site juxtaposition. However, a molecular isomerization should be first order and independent of FLP protein concentration, as is consistent with the 0°C experiment data presented in this study. Thus, the most likely rate-limiting step at 0°C is step 3, isomerization between two forms of a Holliday intermediate-FLP protein complex.

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